

**EXPLORING THE CIRCADIAN OUTPUTS AND FUNCTIONS OF HTP-1  
IN *NEUROSPORA CRASSA***

An Undergraduate Research Scholars Thesis

by

JUSTIN WAYDE VICKERY

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Research Advisor:

Dr. Deborah Bell-Pedersen

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## ABSTRACT

Exploring the circadian outputs and functions of HPT-1 in *N. crassa*. (May 2014)

Justin Wayde Vickery  
Department of Biology  
Texas A&M University

Research Advisor: Dr. Deborah Bell-Pedersen  
Department of Biology

Although many genomes have now been sequenced, genes without a known function still comprise approximately 40% of both the human and *Neurospora crassa* genomes. The ability to control gene expression by both activation and repression is a powerful method to elucidate biological function. This is often accomplished through the use of a gene promoter that can be manipulated to direct expression levels. However, current promoters used in *N. crassa* respond to multiple environmental cues, or only induce expression in one direction (up or down). To aid in the study of genes, we developed a strategy using PCR and homologous recombination to insert the *tcu-1* promoter in front of any gene of interest. The *tcu-1* gene in *N. crassa* encodes a high affinity copper transporter that is tightly controlled by copper availability. Excess copper represses expression, while copper depletion, via the use of a copper chelator, activates expression. This promoter is ideal for gene control studies in that it displays a high level of specificity, and can direct expression in both directions (up and down). The kinetics of induction and repression were found to be rapid, and the effects long lived. To test if copper repression can recapitulate the loss of viability that an essential gene knockout causes, we placed  $P_{tcu-1}$  upstream of the essential gene, *hpt-1*. The addition of excess copper drastically reduced the growth rate as expected. Thus, this strategy will be useful to probe the biological function of any

*N. crassa* gene through controlled expression. Importantly, essential genes can be studied without affecting viability, as would occur if mutated or deleted.

## **DEDICATION**

I would like to dedicate this research to my parents, past and present teachers, coaches, and everyone who has challenged me to be my best. The continual support and encouragement of these people has helped me to reach my goals and chase my dreams.

## **ACKNOWLEDGEMENTS**

I would like to thank the entire Bell-Pedersen lab for their support and encouragement. I owe a special thank you to Dr. Bell-Pedersen and Dr. Teresa Lamb for their guidance and willingness to teach me so much about research. Their teaching and influence has not only shaped me as a student, but as a person.

## NOMENCLATURE

HPT-1	Histidine Phospho-Transferase
OS	Osmotic Stress
RRG	Response Regulator
MAP	Mitogen-Activated Protein
<i>frq</i>	<i>frequency</i> gene
FRQ	FREQUENCY protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
IP	Immuno-precipitation
GFP	Green Fluorescent Protein
LL	Constant Light
DD	Constant Dark

# CHAPTER I

## INTRODUCTION

### **Development of a copper inducible promoter**

Recent developments in the field of genetics have allowed sequencing projects to identify genes in many organisms. Even though the sequencing projects have advanced our understanding in biology, the function of many individual genes is still unknown. It has been estimated that the function of about 40% of both the human and *Neurospora crassa* genomes are still unknown (Galagan et al. 2003; Venter et al. 2001). Even the model eukaryotic organism, *Saccharomyces cerevisiae*, still has approximately 1000 genes that are uncharacterized.

One standard method to explore gene function is to control expression (up or down) and examine the biological responses. A current method of gene control in *N. crassa* is through the manipulation of the *qa-2* promoter. Gene expression is activated in the presence of quinic acid and low sugar concentrations, and expression is repressed at low levels of quinic acid and high sugar concentrations (Campbell et al. 1994; Giles et al. 1985). Although the *qa-2* promoter is useful, it is not ideal due to the defect of being sensitive to the nutritional state.

In addition to the *qa-2* promoter, the copper metallothionein gene promoter (*cmt*) has been used as a copper inducible promoter (Kupper et al. 1990; Schilling et al. 1992), but has not been studied for gene repression. The *vvd* promoter can also be used to drive light-controlled induction of gene expression (Hurley et al. 2012), but faces problems when light-regulated



processes are being studied. Other promoters exist that are able to drive gene expression, but problems arise because they are also controlled by nutritional and/or developmental state.

Copper is an essential cofactor for many processes that occur within the cell. Organisms have developed means to control the uptake of copper from the environment. Studies in *S. cerevisiae* have shown that only a small portion of the genome is regulated by copper availability, making copper an ideal molecule for control of gene expression (Gross et al. 2000; Rustici et al. 2007; van Bakel et al. 2005). A high affinity copper transporter promoter has been successfully used in *Schizosaccharomyces pombe* and *Cryptococcus neoformans* to drive gene expression (Bellemare et al. 2001; Ory et al. 2004). The homologous gene for *N. crassa* - *tcu-1* - was studied as a candidate for controlling expression. The *tcu-1* gene encodes for a high affinity copper transporter.

First, the expression of *tcu-1* was studied and determined to be influenced by external copper levels, and the kinetics of the response to copper availability was defined. Next, the specificity of the *tcu-1* promoter,  $P_{tcu-1}$ , was determined. Finally, a PCR strategy was developed for inserting the *tcu-1* promoter into the 5' region of any target gene and this strategy was confirmed by controlling the expression of the clock oscillator gene, *wc-1*, with external copper levels.

The *tcu-1* promoter will be used to study the function of the histidine phosphos-transferase, HPT-1 in *N. crassa*, an essential gene that is necessary for the osmo-sensing (OS) pathway.

## Known interactions of the clock and osmotic stress pathway

The components of the OS pathway are shown in Figure 1, and the functions of the components are inferred from their homology to the well-studied *S. cerevisiae* HOG pathway (Borkovich et al. 2007). The MAPK module in the OS pathway is similar to the p38 MAPK pathway in mammals, important in cell growth and differentiation (Qi 2005; Schaeffer and Weber 1999).

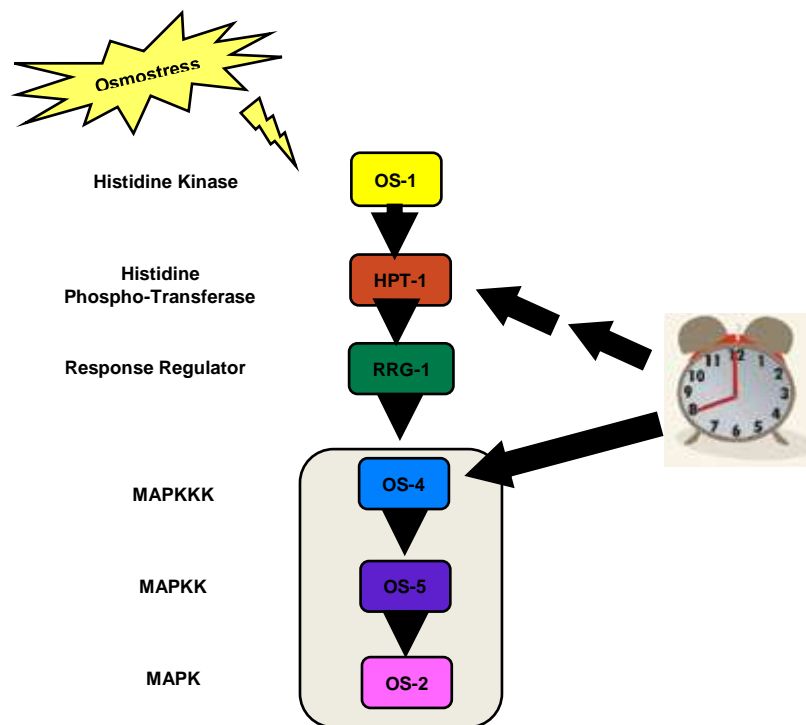


Figure 1: Components of the OS signaling pathway. Arrows to components of the OS pathway represent known direct (single arrow) or indirect (double arrow) connections from the clock.

Under osmotic stress conditions, the osmo-sensor, OS-1, signals through the histidine phospho-transferase, HPT-1, to the response regulator, RRG-1. RRG-1 then affects the kinase activity of the MAP kinase kinase, OS-4, which activates the entire MAP kinase cascade, leading to the

phosphorylation of OS-2. However, in addition to the environmental signaling input to OS-1, we know that the circadian clock regulates this pathway as well.

In the *N. crassa* circadian oscillator (Figure 2), the white collar complex (WCC) functions to receive light input through its photoreceptor domain and to control transcription of the clock gene, *frequency* (*frq*). FRQ protein accumulates and feeds back to inhibit the activity of WCC. Eventually, the FRQ protein is degraded, relieving the repression on WCC, and a new cycle begins. With built in delays, the entire feedback cycle takes approximately one day.

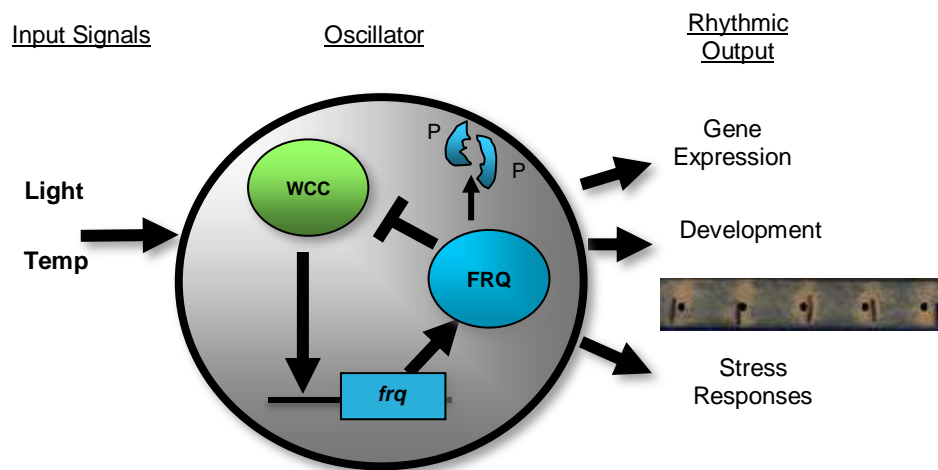


Figure 2: Model of the *Neurospora oscillator*. The components of the clock, described in the text, generate daily rhythms in many cellular processes through clock-control of gene expression.

The WCC controls transcription of *frq*, which is necessary to drive the gears of the oscillator, but the WCC also has other targets that are part of the clock output. Our lab discovered that the WCC binds to the *os-4* promoter to drive rhythmic expression of OS-4 protein (Smith et al, 2010; Lamb et al 2011). We also discovered that there are daily rhythms in the activation state of OS-2 that occur independent of an osmotic stress (Vitalini et al, 2007). Importantly, WCC

binding to the *os-4* promoter is required for rhythmic OS-2 activation (Lamb et al, 2011). Thus, clock input to *os-4* is essential for rhythms in OS-2 activation.

There is another input from the clock to the OS pathway that is not well defined. Rhythms in transcription and protein accumulation of HPT-1 have been observed (Lamb et al, 2011).

However, this input is likely to be indirect given that the WCC does not bind the *hpt-1* promoter (Figure 1). Moreover, we do not know how HPT-1 is rhythmically produced, what it interacts with, what effect altered levels of HPT-1 would have on signaling, or what role it plays in driving rhythms in the OS pathway or other stress pathways.

In order to test the hypothesis that rhythmic HPT-1 is necessary for rhythmic activation of OS-2, we will drive the expression of *hpt-1* in a non-rhythmic way and examine the consequences. The effects of *hpt-1* overexpression and reduced expression will be studied with respect to growth rate, stress signaling, and circadian rhythms in the OS-pathway.

Exactly how HPT-1 performs its function is currently unclear. While HPT-1 is predicted to relay the osmotic stress signal from OS-1 to RRG-1, interactions with these proteins have not been tested. Furthermore, as the sole histidine phospho-transferase, it is predicted to interact with the other 10 histidine kinases found in *N. crassa* (Borkovich et al, 2004). This raises the question of whether rhythms in HPT-1 will affect signaling through the other histidine kinase pathways.

Identifying HPT-1 interacting proteins will help define the functional consequences of HPT-1 rhythms in the OS, and other signaling pathways. Expressing HPT-1 in a non-rhythmic way will help determine the role HPT-1 plays in driving rhythmic output from the OS, and possibly other

pathways. Together these two experimental lines will help define the role HPT-1 in circadian output and stress response pathways.

## CHAPTER II

### METHODS

#### Culture Conditions

*N. crassa* (FGSC #2489, 74-OR23-IV, *mat a*, called “WT”) was grown in 75 mL 1X Vogels salts, 2% glucose, 0.5% arginine, pH 6.0 (unless otherwise noted) shaking cultures inoculated with mycelial discs cut from mats grown in the same media (McCluskey et al. 2010; Vogel 1956). This standard media contains 50  $\mu$ M CuSO<sub>4</sub>. After 24 hr of growth, cultures were treated with CuSO<sub>4</sub> (C7631; Sigma-Aldrich) and/or bathocuproinedisulfonic acid (BCS, B1125; Sigma-Aldrich) as described. The concentration of CuSO<sub>4</sub> indicated in each experiment is the final total concentration. For the specificity experiment, cultures were treated with 10 mM quinic acid (138622; Sigma- Aldrich), 1 mg/mL fludioxonil (46102; Fluka), 0.4 M NaCl, 10 mM hydrogen peroxide (216763; Sigma-Aldrich), 0.05% sodium dodecyl sulfate (SDS, L4390; Sigma-Aldrich), 1X Westergaard’s medium (Westergaard and Mitchell 1947), or placed on a moist paper and exposed to the air.

To examine the effect of P<sub>*tcu-1*</sub>-driven histidine phosphotransferase (HPT-1) on growth rate, race tubes containing 1X Vogels salts, 2% glucose, 0.5% arginine, 50  $\mu$ g/mL biotin, and 1.5% agar with CuSO<sub>4</sub> or BCS added before autoclaving were inoculated with ~7-day old conidia and grown for 3 d at 30°C in constant light (LL).

### Construction of the pCR blunt *bar::P<sub>tcu-1</sub>* plasmid

The *Streptomyces hygroscopicus bar* gene conferring resistance to glufosinate (Avalos et al. 1989) was amplified by polymerase chain reaction (PCR) from pBAR-GEM 7.2 (Pall and Brunelli 1993) using primers Bar-BstB1-F (5' TTCGAAGTCGACAGAAGATGATATTG 3') and Bar-BstB1-R (5' TTCGAAGAACCGGCAGGCTGAAGTCC 3'). The resulting 912-bp DNA fragment was ligated into pCR blunt (Invitrogen, Carlsbad, CA) generating plasmid pJV1. The 1690-bp DNA fragment containing the *tcu-1* promoter was generated by PCR on wild-type (WT) genomic DNA using primers *P<sub>tcu-1</sub>* F-NotI (5' TTTGCGGCCGCGATGGGATAGAGAGAATGGC 3') and *P<sub>tcu-1</sub>* R *Ap*I (5' TTTGGGCCCCGTTGGGGATGTGTGTGC 3'). The PCR product was cut with NotI and ApI, and ligated into pJV1 digested with the same enzymes, creating pCR blunt *bar::P<sub>tcu-1</sub>* (plasmid and sequence deposited at the Fungal Genetic Stock Center).

### Strains

To generate *P<sub>tcu-1</sub>*-driven HPT-1 cell line, 5' and 3' integrating fragments of DNA were constructed as described in the Results, and ~50 ng of each were transformed by electroporation into DBP 1202 (*ras-I<sup>bd</sup>*, *mat a*, *hpt-1::FLAG::hph*, *rrg-1::HA::hph*, called *P<sub>hpt-1</sub>hpt-1*) strain, (McCluskey et al. 2010). For *hpt-1*, the dual-tagged strain was generated for future studies to investigate interactions between HPT-1 and response regulator-1 (RRG-1). Colonies were selected on 250 µg/mL glufosinate ammonium (G596950; Toronto Research Chemicals)-containing transformation plates.

### **Protein extraction and Western blots**

*N. crassa* tissue was blotted dry, frozen, and subsequently ground under liquid nitrogen. Proteins were extracted from ground tissue (Garceau et al. 1997), and 100 µg of total protein was run on standard SDS polyacrylamide electrophoresis gels, and electro-transferred to polyvinylidene difluoride membranes (IPVH00010; Millipore). HPT-1-FLAG was bound with primary DYKDDDDK Tag Antibody (diluted 1:1000, 2368; Cell Signaling Technology), recognized with goat anti-rabbit horseradish peroxidase-conjugated antibody (diluted 1:20,000, 170-6515, Bio Rad), and visualized with a chemiluminescence solution (1.25 mM Luminol [A8511; Sigma-Aldrich], 0.2 mM p-coumaric acid [C9008; Sigma-Aldrich], 100 mM Tris-Cl, pH 8.5, and freshly added hydrogen peroxide [diluted 1:3333, 216763; Sigma-Aldrich]). After detecting via Western blotting, membranes were stained with an amido black solution (0.1% amido black [N3393; Sigma-Aldrich], 10% acetic acid, 25% isopropanol) to reveal all proteins as an indication of protein transfer.

### **RNA extraction and Northern blots**

RNA was extracted and purified from ground tissue, and 10 µg were run on denaturing formaldehyde gels (Bell-Pedersen et al. 1996). Gels were blotted to Nitro Pure membranes (#WP4HY00010; GE), and hybridized with gene specific RNA-probes labeled with [ $\alpha$ -<sup>32</sup>P]-UTP (BLU507H250UC; PerkinElmer).



## CHAPTER III

### RESULTS

#### *tcu-1* expression is controlled by copper availability

To test if the high-affinity copper transporter gene *tcu-1* is regulated by copper availability, WT *N. crassa* was grown in liquid media with copper sulfate (50-250  $\mu$ M), or a copper chelator (BCS, 50-250  $\mu$ M) and harvested after 8 hr. Levels of *tcu-1* mRNA were analyzed by Northern blot (Figure 3). Excess copper turned down *tcu-1* expression, whereas increasing concentrations of the copper chelator, BCS, induced *tcu-1* expression, as predicted (Korripally et al. 2010).

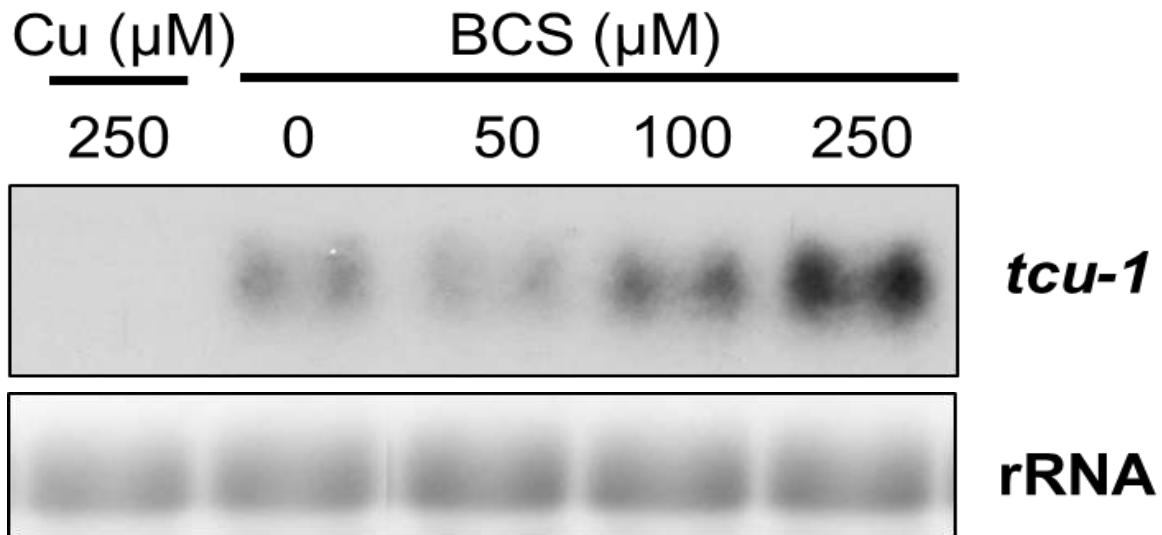


Figure 3: Northern analysis showing levels of *tcu-1* mRNA. No expression is seen when cells are grown in copper. However, the levels of *tcu-1* expression increase with increasing concentrations of BCS. rRNA shows even loading.

## The kinetics of *tcu-1* expression

Expression of *tcu-1* was determined over a series of time points after either the addition of 50  $\mu$ M or 250  $\mu$ M BCS. Significant induction of *tcu-1* occurred after 4 hrs in 50  $\mu$ M BCS and after 2 hrs in 250  $\mu$ M BCS (Figure 4). Maximal levels of induction occurred between 8 and 24 hr, and expression remained high at 24 hr after addition of either concentration of BCS.



Figure 4: Expression of *tcu-1* mRNA in 50  $\mu$ M and 250  $\mu$ M BCS, with induction occurring at 2 hrs and 4 hrs, respectively. rRNA shows even loading.

To test gene turn down/off, *tcu-1* expression was first activated with either 50  $\mu$ M BCS or 250  $\mu$ M BCS for 4 hrs.  $\text{CuSO}_4$  was then added at 200  $\mu$ M and 100  $\mu$ M, respectively, and the effect analyzed by Northern blot. Figure 5 shows rapid turn off in both experiments with nearly complete repression by 0.25 hr and remaining low for at least 6 hrs.

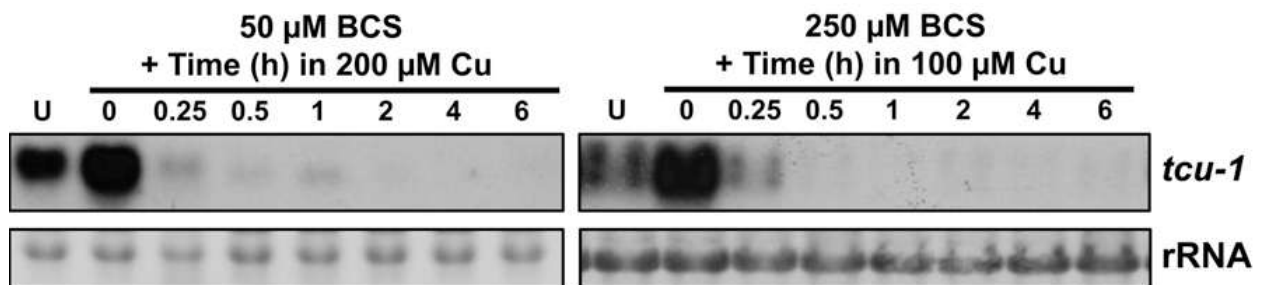


Figure 5: Levels of *tcu-1* mRNA were examined in untreated (U) or BCS treated (either 50 or 250  $\mu$ M) cultures that were subsequently treated with 200 or 100  $\mu$ M  $\text{CuSO}_4$ , respectively, for the indicated times by Northern analysis.

### **Copper control of *tcu-1* is highly specific**

Specificity is an important quality for a controllable gene promoter. Ideally, the promoter would respond only to a specific signal (in this case copper) and not to other external factors.

To determine the specificity of the *tcu-1* promoter, we tested the effects of media composition and various stresses on the expression of wild type *tcu-1* under its native promoter. Figure 5 shows that in 0.01% glucose growth conditions, *tcu-1* is induced by BCS. Weak induction occurs by quinic acid, but this can be explained by the acidic environment inhibiting copper import, thus simulating low copper availability (Blackwell et al. 1995). In 2% glucose growth conditions, *tcu-1* is strongly induced by BCS and only weakly affected by the other stresses applied. Loading of the RNA samples was uniform as shown in the bottom panel, with the exceptions that the detergent treated samples were somewhat degraded and the Westergaard's growth sample had lower levels of total RNA. The data shows that *tcu-1* is highly specific to copper availability and relatively unaffected by other stresses. Previous studies have also shown that *tcu-1* expression is independent of glucose, light, or circadian clock control (Lewis et al. 2002; Smith et al. 2010; Xie et al. 2004).

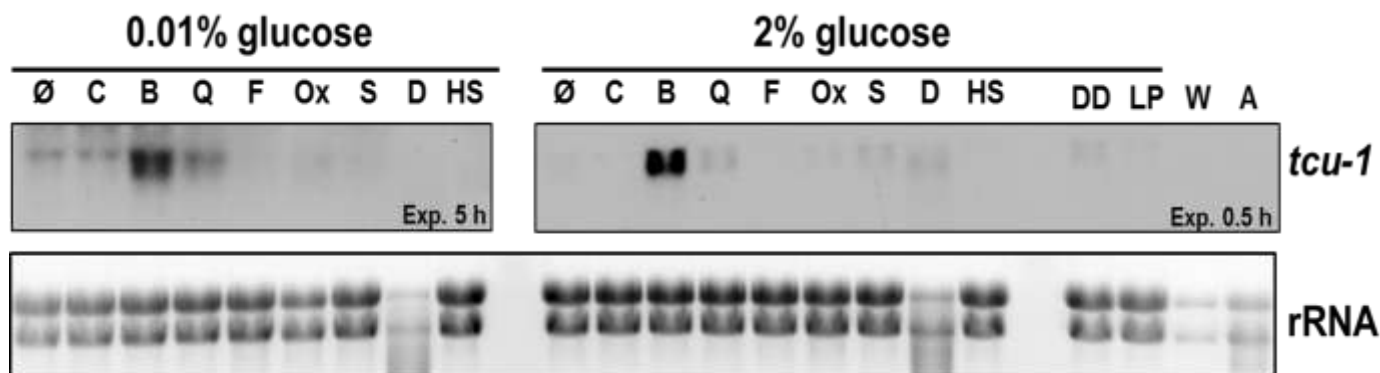


Figure 6: Effects of stress and nutrition on *tcu-1* gene expression in a WT strain. The levels of mRNA were examined by Northern analysis in control untreated (Ø), 250  $\mu$ M CuSO<sub>4</sub> (C), 250  $\mu$ M BCS (B), 10 mM quinic acid (Q), 1  $\mu$ g/mL fludioxonil (F), 10 mM H<sub>2</sub>O<sub>2</sub> (oxidative stress, X), 0.4M NaCl (salt, S), 0.05% SDS (detergent, D), and 30 min 42°C heat shock (HS) in 0.01% and 2% glucose conditions, as well as in 24-hr DD, a 30 min-light pulse (LP), Westergaard's medium (W), and air exposure (A). Ethidium bromide staining of the rRNA is shown in the bottom panel.

### PCR strategy to integrate the *tcu-1* promoter in front of a target gene

Figure 7 shows the strategy designed to integrate  $P_{tcu-1}$  in front of any desired gene.

Construction of each PCR fragment is accomplished through a two-step process. Primers P1 and P2 are used to amplify 500 bp of the 5' flank of the target gene from genomic DNA creating PCR1. Primers P3 and P4 are then used to amplify the 3' end of the *bar* gene from the plasmid pCR blunt *bar*  $P_{tcu-1}$  creating PCR2. These two fragments are then used with P1 and P2 to create the 5' integration fragment. Primers P5 and P6 are then used to amplify the 5' end of the *bar* gene fused to the *tcu-1* promoter using the plasmid pCRblunt *bar*  $P_{tcu-1}$  as the template creating PCR4. PCR5 is made using P7 and P8 on genomic DNA to amplify 500 bp of the target gene ORF. The 3' integration fragment is constructed using P5 and P8 on PCR4 and PCR5 templates.

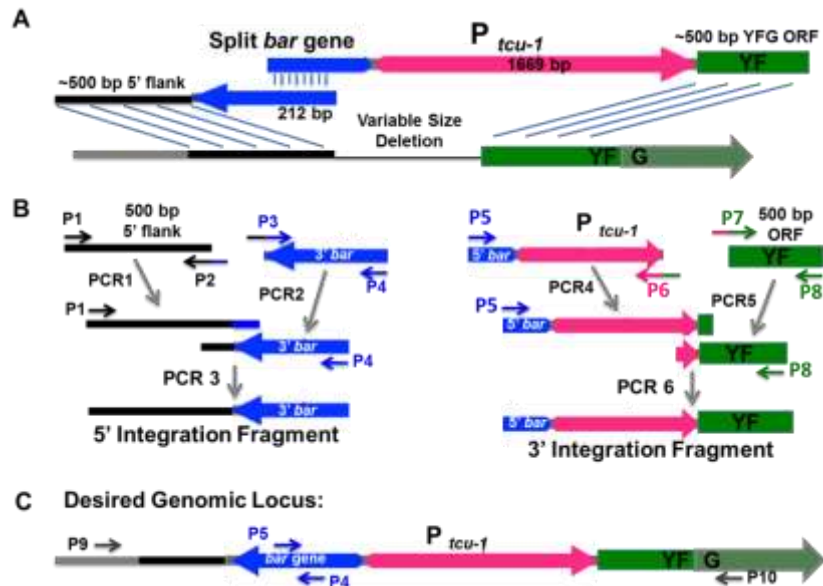


Figure 7: Scheme for integrating  $P_{tcu-1}$  to a target genomic locus. (A) An overview of the scheme showing the integration of two PCR fragments via homologous recombination. (B) Production of the two integration fragments. (C) Final genomic locus.

### Copper control of the essential gene *hpt-1*

In order to test the control of a gene under the *tcu-1* promoter, we chose to study the essential *hpt-1* gene. We inserted  $P_{tcu-1}$  into the 5' region of *hpt-1* using the PCR method described.

Figure 8 shows the protein levels of *hpt-1* under both the native promoter and the copper-driven promoter. A FLAG tagged strain was used in the Western analysis. Expression was induced with BCS, and then treated with 250  $\mu$ M  $\text{CuSO}_4$  for varying times. Protein expression under the native promoter was unaffected by the addition of copper. HPT-1 protein levels under the copper promoter were initially increased at time 0 through the addition of BCS. However, the levels gradually decreased and were undetectable after 6 hrs in  $\text{CuSO}_4$ .

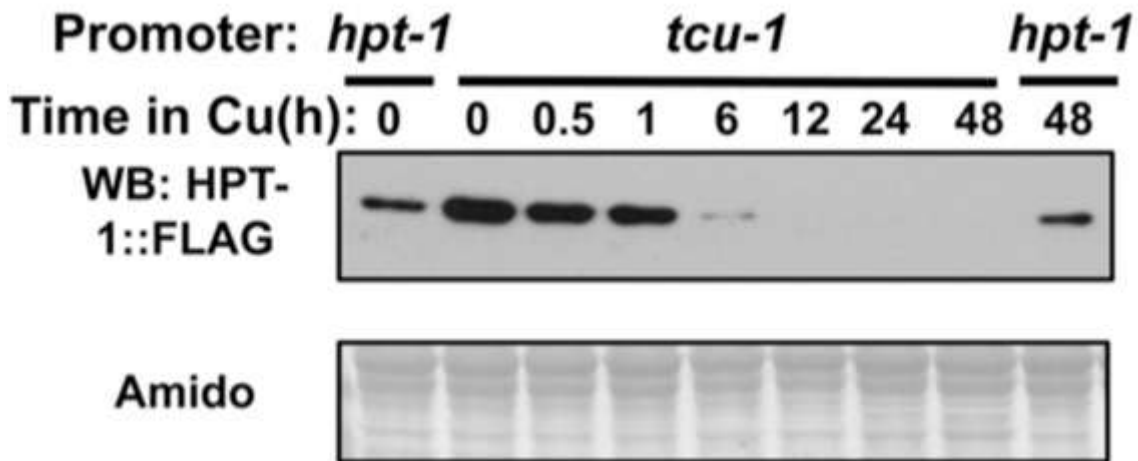


Figure 8: Western analysis showing protein levels of *hpt-1* under the control of the native promoter and the *tcu-1* promoter. Amido staining of total protein showed even loading.

Because *hpt-1* is an essential gene, a deletion will affect the viability of the organism. In an attempt to phenocopy an *hpt-1* deletion, race tubes containing  $\text{CuSO}_4$  and/or BCS were inoculated with a strain containing *hpt-1* under its native promoter ( $P_{hpt-1}hpt-1$ ) and a strain containing *hpt-1* under control of the copper promoter ( $P_{tcu-1}hpt-1$ ). The growth rate of *hpt-1* under its native promoter was unaffected by the copper/BCS content of the race tubes, while the growth rate of  $P_{tcu-1}hpt-1$  was strongly inhibited by increasing copper concentration (Figure 9).

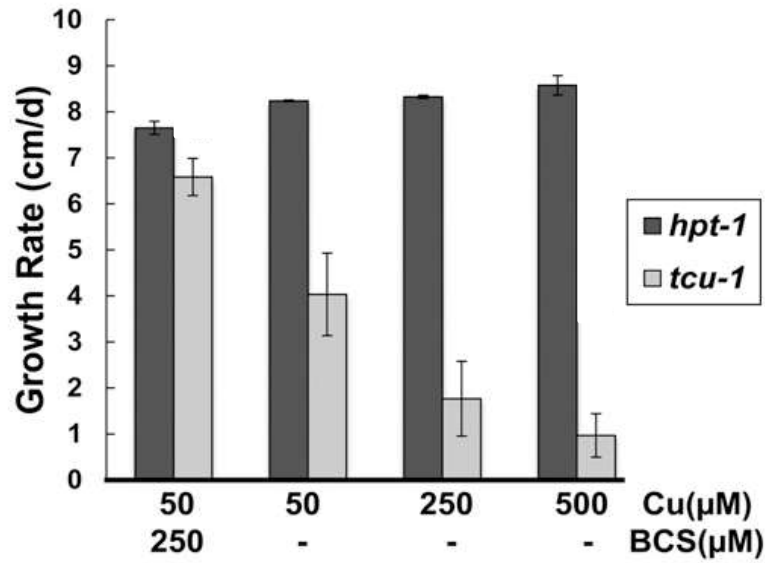


Figure 9: Growth rate comparison when *hpt-1* is under its native promoter versus under the control of the *tcu-1* promoter.

### Search for HPT-1 protein interactions

In order to identify potential HPT-1 interacting proteins, the first step was to perform an immunoprecipitation (IP) using HPT-1::Flag to determine if the epitope tagged protein could be isolated. The IP was performed using the strain DBP 1202 ( $P_{hpt-1}hpt-1$ ) and DBP 1660 ( $P_{tcu-1}hpt-1$ ) under normal growth conditions (V2G) and in the presence of BCS. Figure 10 shows the Western analysis of both the protein inputs and the IP. Compared to normal growth, there is an enhancement in HPT-1 protein levels in the copper-controlled strain in the presence of BCS. However, the IP was unable to clearly pull down significant levels of FLAG-tagged HPT-1 protein, even after the induction by the addition of BCS. In addition, we looked for associating proteins by Coomassie stain, but failed to find any specific interactions.

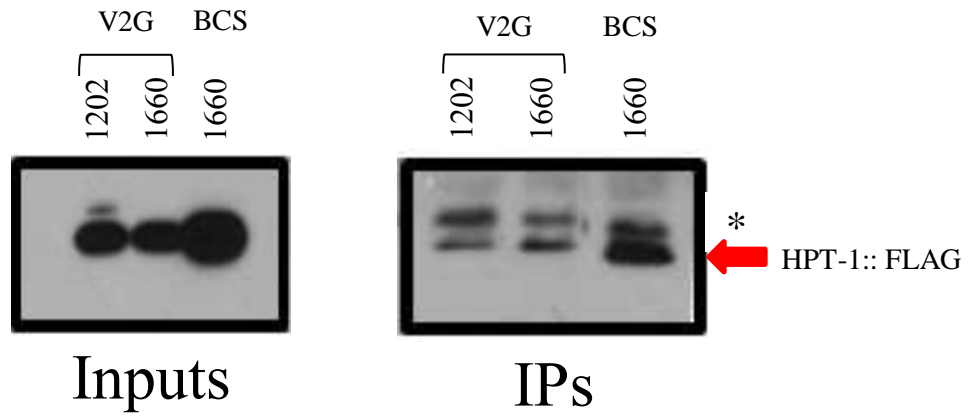


Figure 10: Western analysis of HPT-1 inputs and IPs. Inputs represent 100μg of loaded protein. IPs are from 2mg of protein. Induction is seen in the presence of BCS, however enhancement/pull down from IP is not significant. \* Upper band in IP represents non-specific cross reacting proteins.



## CHAPTER IV

### CONCLUSION

The *tcu-1* promoter was identified as a candidate for a controllable promoter based on its similarity to the *ctr-4* promoter in *S. pombe* and *C. neoformans*. We characterized the expression of this gene, confirming that it is responsive to copper availability. In addition, the response kinetics were studied, and it was found that gene expression occurs within 2 to 4 hrs after the addition of the copper chelator BCS, and repression is rapid with the addition of CuSO<sub>4</sub>. Ideally, a controllable gene promoter is highly specific to one signal. We found that the *tcu-1* promoter responds specifically to copper availability, making it an ideal controllable promoter for studying the functions of unknown genes and essential genes.

A system for integrating P<sub>*tcu-1*</sub> in front of a desired gene was developed that relied on PCR and homologous recombination. This system is advantageous in that only a single strain needs to be generated for both overexpression and repression.

We successfully used this system to integrate the copper promoter in front of the essential gene *hpt-1*. Overexpression was every effective and repression with the addition of copper phenocopied the deletion strain.

While essential genes have been difficult to study using mutations and knockouts, this system allows for the flexible control of gene expression. Thus essential genes can be examined simply by tuning the levels of expression up or down based on copper availability.

Initial experiments directed at purifying the HPT-1 protein to identify what HPT-1 interacts with involving immunoprecipitation of FLAG-tagged HPT-1 and Western analysis have proven difficult. A new strategy is currently being tested where HPT-1 will be tagged with GFP rather than FLAG. This will allow to the use of GFP-specific nanobodies for the immunoprecipitation. While normal antibodies consist of a heavy and light chain, nanobodies are composed of a single chain, making them considerably smaller. This reduces the potential for cross binding, resulting in less background protein, and enhances binding specificity to GFP (Neumüller et al. 2012).

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